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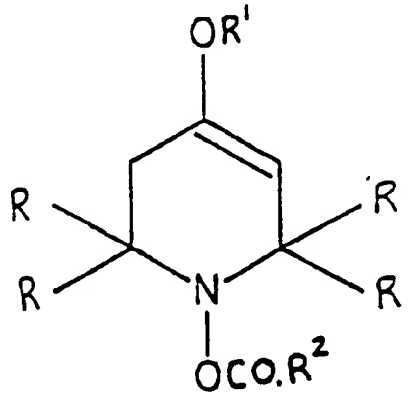

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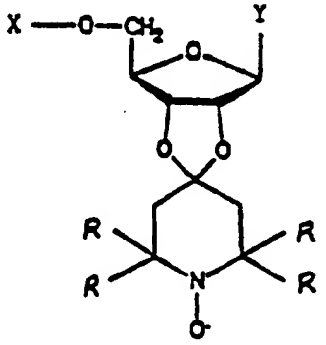

(2)

(57) Abstract

Compounds of formulae (1), wherein R represents a CH_3 or CD_3 group; R^1 represents an alkyl group; R^2 represents an alkyl or aryl group; and N may be either an ^{14}N or ^{15}N atom, with the proviso that when R represents CD_3, the methylene and vinyl hydrogen atoms of the six-membered ring are deuterium; and (2), wherein R and N are as defined above, X represents hydrogen or a mono-, di- or triphosphate group, or a phosphate ester derivative; and Y represents a purine or pyrimidine base, with the proviso that when R represents CD_3, the methylene hydrogen atoms of the six-membered piperidine ring are hydrogen or deuterium. Processes for the preparation of these compounds are also described. The spin labelled nucleotide and nucleoside compounds are suitable for use as probes, for example, in protein structure and orientation studies.

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SYNTHESIS AND USES OF SPIN LABELLED RIBONUCLEOSIDES AND RIBONUCLEOTIDES

This invention relates to the preparation of spin labelled compounds from novel precursors and their use as probes. More specifically, the invention provides conformationally restricted spin labelled ribonucleosides and ribonucleotides which are useful, for example, in protein orientation studies.

Spin labelled probes have proved useful in dynamic and orientation studies of proteins using electron spin resonance (ESR), such as monitoring the movement of protein cross-bridges during muscle contraction. A significant problem with existing spin labelled nucleotide probes, however, is that the spin label moiety is mobile and thus does not give accurate information about the position of the nucleotide probe on the protein which is under investigation.

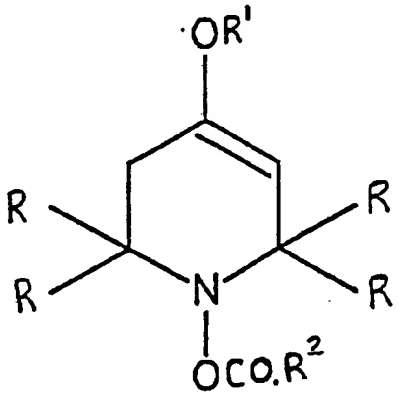
The use of spin label molecules in nuclear magnetic resonance (NMR) work is also well known. Their presence in labelled probes produces characteristic line broadening effects in the NMR spectra which can give important information during, for example, structural studies of a variety of biological macromolecules.

A number of reactions to produce various derivatives of nucleosides or nucleotides have previously been described. Hampton, J. Am. Chem. Soc., 83, 3640 (1961); Hampton et al., J. Am. Chem. Soc., 87, 5481 (1965); Reese et al., Tetrahedron, 26, 1023 (1970) and Chladek & Smrt, Coll. Czech. Chem. Commun., 28, 1301 (1963) illustrate a range of reactions that

essentially involve the acid-catalysed additions of ketones, ketals or enol ethers to the vicinal diol of the ribose ring. Grindley et al., Carbohydrate Res., 140, 215 (1985) describe the use of 1-ethoxycyclohexene and 2-methoxypropene to form cyclic ketals of a range of non-ribose sugars. Hai et al., J. Med. Chem., 25, 806 (1982) disclose the direct ketalisation of a nucleotide compound.

There is a need for spin labelled nucleoside and nucleotide analogues which overcome the aforementioned disadvantage of existing labelled probes. The present invention aims to provide such compounds.

According to the present invention there are provided compounds of the formula:-



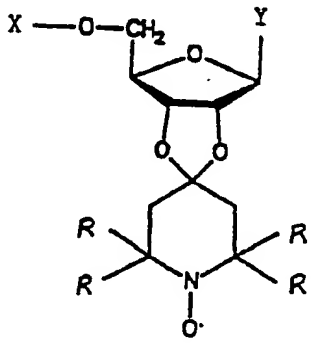
(1)

wherein R represents a CH_3 or CD_3 group;
R^1 represents an alkyl group;
R^2 represents an alkyl or aryl group; and
N may be either an ^{14}N or ^{15}N atom,
with the proviso that when R represents CD_3, the methylene and vinyl hydrogen atoms of the six-membered ring are deuterium.

In principle, R^1 may be any alkyl group and R^2 may be any alkyl or aryl group. It will be appreciated, however, that the presence of relatively small alkyl and aryl groups is usually to be preferred.

These compounds are useful as precursors of spin labelled nucleoside and nucleotide analogues. They have been found to be capable of reacting with ribonucleosides and ribonucleotides to produce spiroketal compounds that, on further modification, lead readily to spin labelled compounds suitable for use as probes.

According to a further embodiment of the present invention there is provided a spin labelled compound of the formula:-



(2)

wherein R is as defined above;

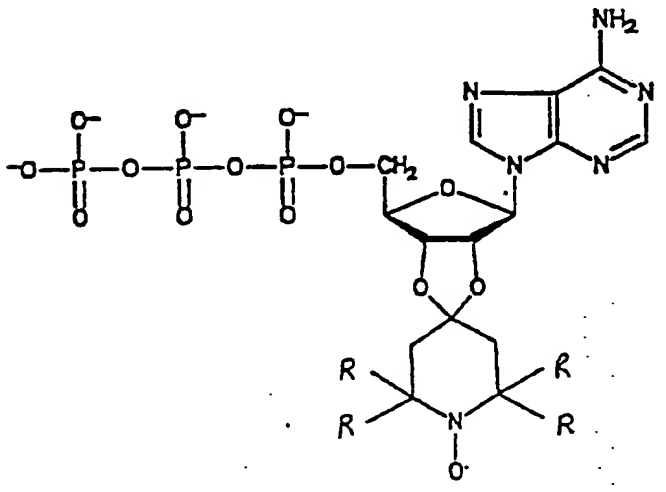
X represents hydrogen, a mono-, di- or triphosphate group, or a phosphate ester derivative; and

Y represents a purine or pyrimidine base, with the further proviso that when R represents CD_3, the methylene hydrogen atoms of the six-membered piperidine ring are hydrogen or deuterium.

If desired, either of X or Y may be radiolabelled. Examples of group X as a phosphate ester derivative include the 3-thiotriphosphate, the P^3-1-(2-nitrophenyl)ethyl group or an oligonucleotide that would produce spin labelled derivatives of ATP(γS), caged ATP or an oligonucleotide.

As will be readily appreciated, the spin labelled compound (2) incorporates the skeleton of the aforementioned type of compound (1).

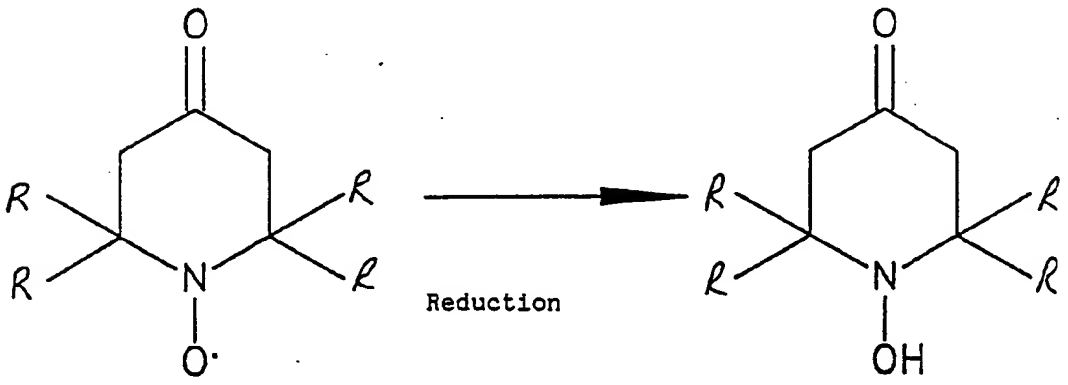
The spin labelled nucleotide and nucleoside compounds of this invention may comprise either a purine or a pyrimidine base. Thus, in the above formula, group Y can represent adenine, guanine, cytosine, uracil, thymine or 5-methylcytosine. A particularly preferred spin labelled compound of the invention has the following formula (wherein X is triphosphate, Y is adenine and R is as defined above):-



(3)

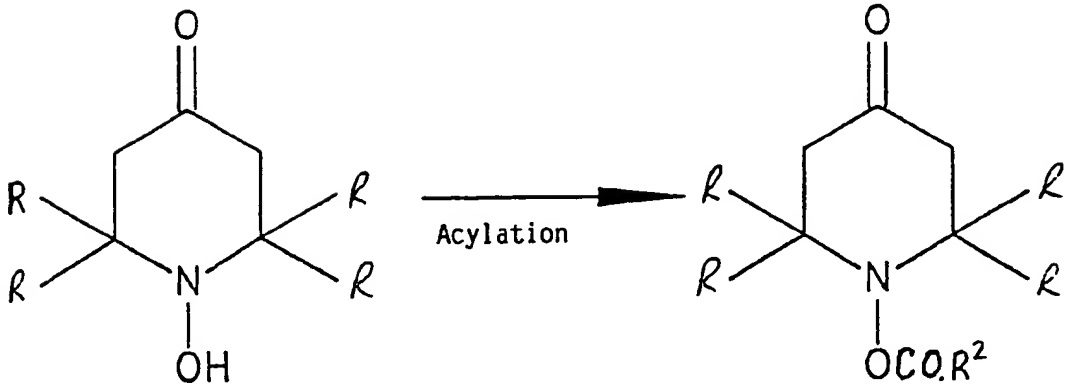
According to the present invention there is further provided a method for preparing a precursor compound (1) of the type defined above and which comprises the following sequence of reactions:-

i)



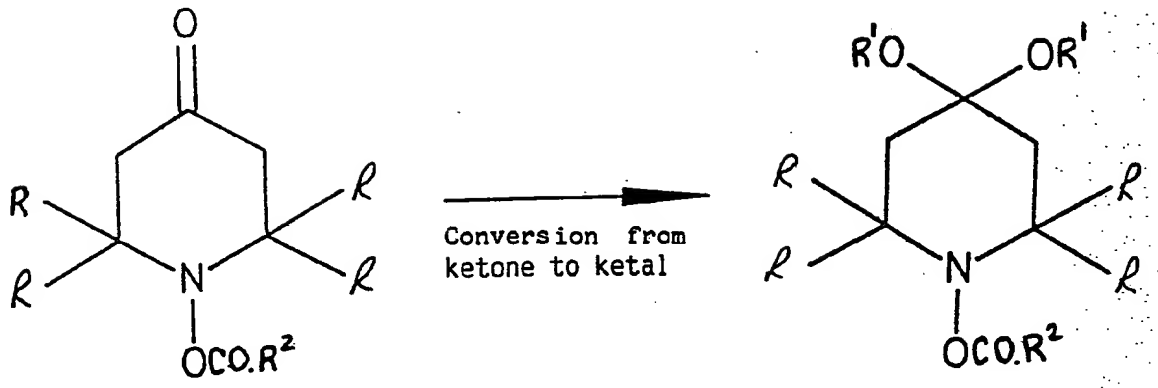
(4) (5)

ii)



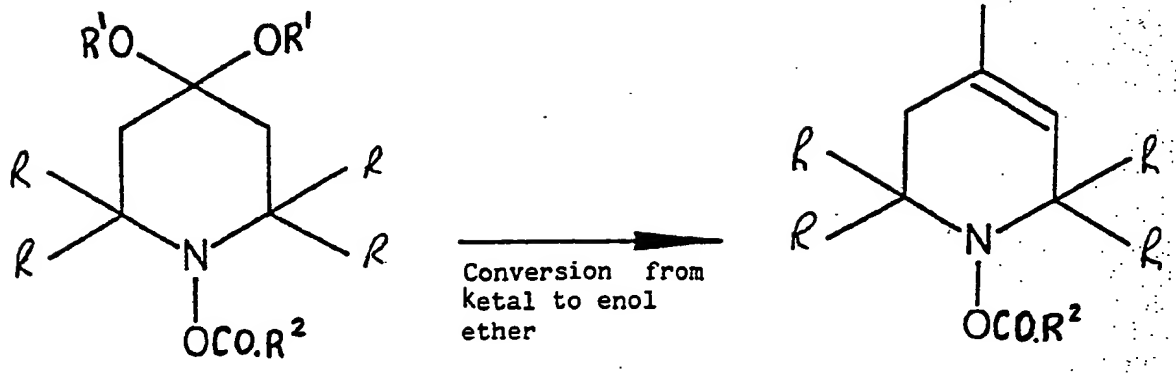
(5) (6)

iii)


O
R
R
R
R
N
OCO.R²
Conversion from ketone to ketal
R'O
OR'
R
R
R
R
N
OCO.R²

(6) (7)

iv)

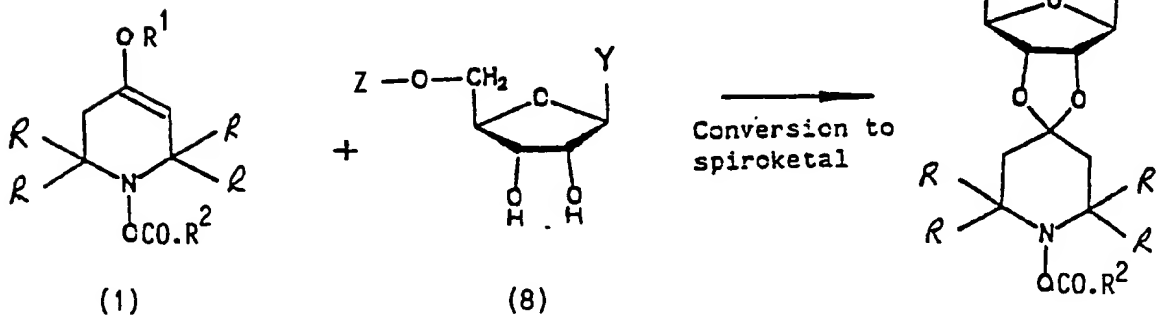

R'O
OR'
R
R
R
R
N
OCO.R²
Conversion from ketal to enol ether
OR'
R
R
R
R
N
OCO.R²

(7) (1)

Steps i), ii) and iii) involve relatively conventional chemistry, so that suitable reactants and process conditions will be readily appreciated by those skilled in the art. The initial reduction of step i) may be performed, for example, in the presence of ascorbic acid. The product is acylated in step ii), for example using acetic anhydride, and then converted in step iii) to a ketal. Finally, in step iv) the ketal is converted to an enol ether. This latter product is the desired precursor compound (1) suitable for use in the synthesis of the spin labelled compounds. Step (iv) proceeds with unexpected facility, because the unfavourable 1,3-diaxial interactions present in the ketal are partially relieved in the enol ether. Steps i) to iv) are illustrated in Example 1 below.

According to the present invention there is still further provided a method for preparing a spin labelled compound, involving the precursor compound (1) of the aforementioned type, which comprises the following sequences of reactions:-

v)



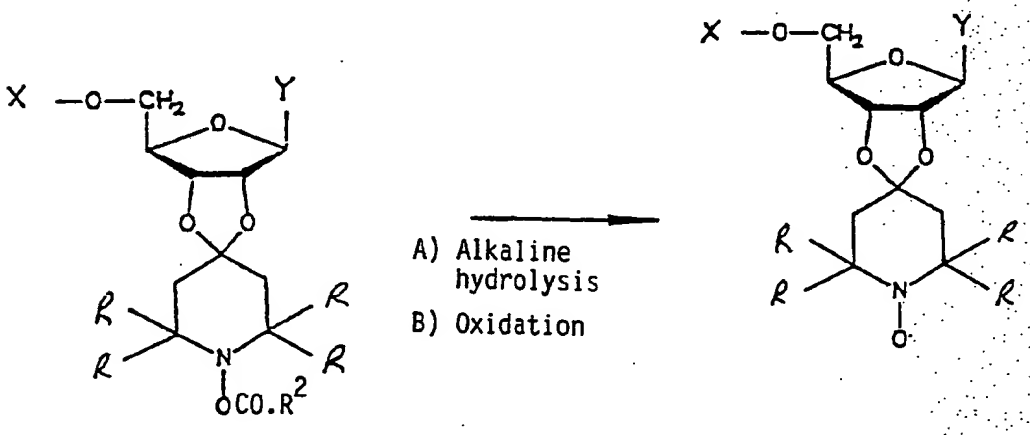
(1) (8) (9)

wherein Z is a protecting group for the 5'-hydroxyl position and which may either equate to group X (as hereinbefore defined, except when X represents hydrogen) or be capable of conversion or removal to leave a group X in that position; and

R, R^1, R^2 and Y are as previously defined, with the further proviso that when R represents CD_3, the 2'- and 3'- hydroxyl hydrogen atoms in compound (8) may be deuterium in order that all four methylene hydrogen atoms of the six-membered piperidine ring of compound (9) are deuterium;

vi) either a) where group Z equates to group X, optionally further phosphorylating the compound (9) at that position, or b) treating the compound (9) such that Z is removed, and optionally phosphorylating, to leave a group X at that position; and

vii)



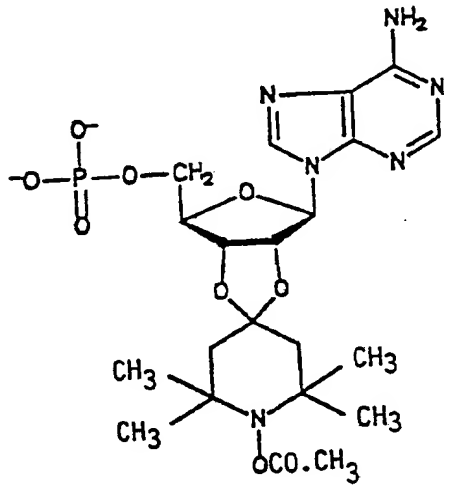
(9a) (2)

It will be appreciated that the route followed in step vi) will depend upon the nature of group Z. Examples of the group Z, when Z does not equate with X, include acyl (R^3CO^-, where R^3 may be alkyl, alkenyl, cycloalkyl or aryl), alkoxycarbonyl (R^3OCO^-, where R^3 is as defined above), allyl or substituted benzyl, such as p-methoxybenzyl, o-nitrobenzyl or 1-(2-nitrophenyl)ethyl. Z may be a group that is retained in the final product, such as a phosphate (as in Example 2 below), or a group that is subsequently removed, such as an acyl group (as in Example 3 below). In any event, step vi) has the effect of converting group Z to group X.

In compound (9) when protecting group Z is removed by alkaline hydrolysis (i.e. is acyl or alkoxycarbonyl), isolation of compound (9a), where X is hydrogen, is still possible because the protecting group is more susceptible to alkaline hydrolysis than the N-acyloxy group that is removed in step vii).

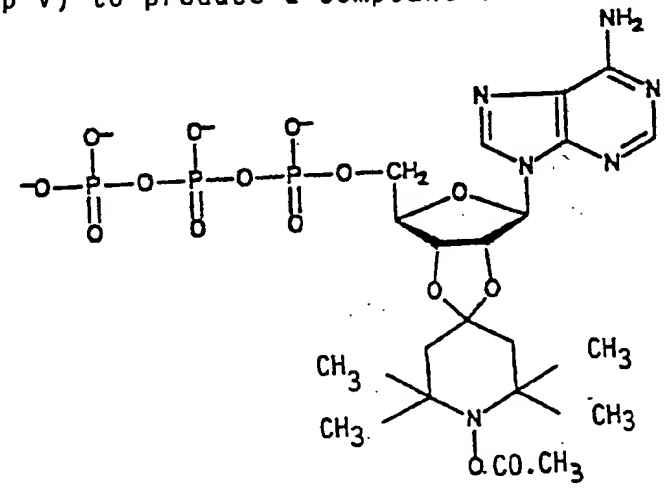
The purpose of the above sequences of reactions is to condense compound (1) with a ribonucleotide or ribonucleoside and then to produce a spin labelled compound (2) which can be used as a probe in ESR or NMR work. The initial conversion to a spiroketal (9) is followed by an optional phosphorylation or further phosphorylation, or a treatment such as hydrolysis, using conventional techniques. Subsequently, the compound is subjected to alkaline hydrolysis of the N-acyloxy group and oxidation to produce the spin labelled compound (2) of the invention.

In a reaction to produce a particularly preferred spin labelled compound of this invention, step v) comprises reacting a precursor compound (1), where R, R^1 and R^2 are each CH_3, with adenosine 5'-monophosphate (AMP) to produce a compound of the formula:-



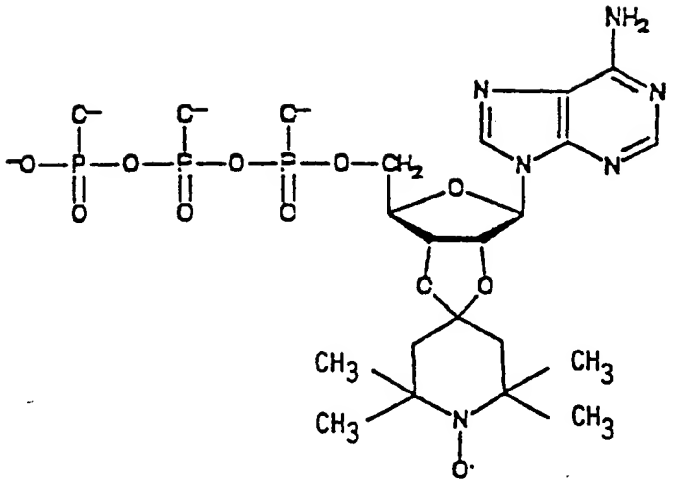
(10)

step vi) comprises pyrophosphorylating the product of step v) to produce a compound of the formula:-



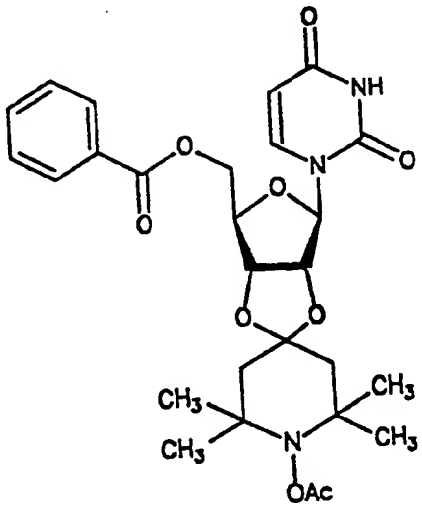
(11)

the step vii) comprises deacetylation and air oxidation of the product of step vi) to produce a compound of the formula:-



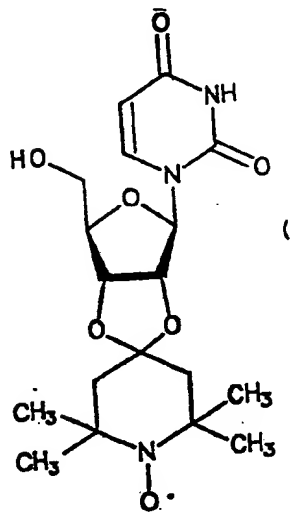
(12)

As an example to illustrate the applicability of the procedure to ribonucleosides, 5'-O-benzoyluridine is reacted with a precursor compound (1) as in step v) to produce compound (13):-



(13)

In steps vi) and vii) the benzoyl and acetyl groups are sequentially removed and the resulting N-hydroxy compound oxidized in air to produce a compound of the formula (14):-



(14)

Substantial difficulties were encountered in the construction of precursors to the desired spin labelled compounds. Conventional acid catalysed condensations (as referenced above) of the ketone (6) or ketal (7) with ribonucleosides and ribonucleotides failed and even the enol ether (1) was substantially less reactive than the less sterically compressed 1-ethoxycyclohexene (the compound used by Grindley et al., see above). This lack of reactivity is accounted for by unfavourable 1,3-diaxial interactions which are created when an alcohol group adds to the enol ether (1) and the further element of ring strain when the spiroketal (9) is formed. A further surprising feature of the condensation is the fact that it only occurs in essentially non-polar solvents. Even the presence of 10% dimethylformamide in the reaction mixture

inhibits the reaction almost completely. This makes for considerable difficulty because of the almost total insolubility of ribonucleotides in non-polar solvents. Finally the purine series was difficult to synthesize because of the lability of the purine-ribose bond under the acid-catalyzed conditions of the synthesis.

Both Broensted-Lowry and Lewis acids were effective in the catalysis of the condensation reactions [step v)]. Overall p-toluenesulphonic acid was found to be the most satisfactory acid catalyst.

A range of spin labelled compounds for use as probes, or as a part of such probes, can be derived from the precursor compound (1) of the present invention. A particular benefit is that the spin label is rigidly attached to nucleotide or nucleoside sugar residues and thereby, for example, rigidly oriented on proteins. This tight coupling of the spin label avoids the previously mentioned disadvantage of mobile nucleotide labels and thus opens up the possibility of new and improved orientation or structure studies of certain proteins. For instance, the use of such probes should enable betterinvestigation of how muscle cross-bridges move during contraction and relaxation, or how DNA interacts with DNA-binding proteins that control gene expression.

The spin label precursor compounds (1) of this invention are suitable for use with all ribonucleotide and ribonucleoside types and thus, for example, could be incorporated into 5'-O-acylated derivatives of

adenosine, cytidine, guanosine, or uridine, or with AMP, CMP, GMP or UMP. It is further envisaged that the spin label precursor compounds (1) could be used to produce other types of labelled probes based on, for example, the common ribonucleoside 5' di- and 5'-triphosphates, caged ATP compounds (i.e. photolabile derivatives of ATP from which ATP can be readily regenerated), pyridine nucleotides (e.g. NAD^+, NADH), ribonucleotide analogues and oligonucleotides.

The compounds and methods of this invention will now be further illustrated by reference to the following Examples. Example 1 relates to the preparation of a spin label precursor compound; Example 2 relates to the introduction of that molecule into AMP and further elaboration to produce a labelled compound suitable for use as a probe. Example 3 relates to the introduction of a spin label precursor into a typical ribonucleoside derivative, 5'-O-benzoyluridine.

EXAMPLE 1

N-Acetoxy-2,2,6,6-tetramethyl-4-piperidone (6). 4-Oxo-2,2,6,6-tetramethylpiperidin-1-oxyl (20g, 188 mmol) was melted by gentle warming and treated with a solution of L-ascorbic acid[1] (37.6g, 190 mmol) in water (320 ml). The solution was stirred vigorously at ambient temperature for 5 min, during which its colour changed rapidly from dark red to pale yellow. It was then diluted with saturated aqueous $NaHCO_3$ (800 ml) in a 5 litre conical flask and cooled in ice. Acetic anhydride (64 ml, 679 mmol) was added over 2 min to the stirred mixture (pH 8). Portions of solid $NaHCO_3$ were added carefully to maintain the mixture at pH 8 until no further pH change occurred (ca. 1 h) and the mixture was then extracted with $CHCl_3$ (3 x 200 ml). The combined $CHCl_3$ extract was washed with saturated aqueous $NaHCO_3$ (2 x 200 ml), dried (Na_2SO_4) and evaporated under reduced pressure, to leave the ketoacetate (6) as a pale solid (23.3g, 94%). A sample recrystallised from petroleum ether gave pale needles, m.p. 95-95.5°C. Anal: Calc. for $C_{11}H_{19}NO_3$: C, 61.9; H, 9.0; N, 6.6; M.W 213.1365. Found: C, 62.0; H, 8.6; N, 6.6; M^+ 213.1362.

N-Acetoxy-4,4-dimethoxy-2,2,6,6-tetramethylpiperidine (7). The crude ketoacetate (6) (23.5g, 110 mmol) and toluenesulphonic acid monohydrate (2.1g, 11 mmol) were dissolved in a mixture of methanol (250 ml) and trimethyl orthoformate (250 ml) and the solution was heated under reflux for 2 h, then cooled to room temperature. 3% Aqueous $NaHCO_3$ (750 ml) was added and the mixture was extracted with $CHCl_3$ (3 x 200 ml). The combined $CHCl_3$ extract was dried (Na_2SO_4) and evaporated under reduced pressure. Distillation of the residual oil afforded the ketal (7) as a pale liquid (25.9g; 91%), b.p. 84°C (0.5 mm Hg). Anal: Calc. for $C_{13}H_{25}NO_4$: M.W. 259.1784. Found: M^+ 259.1770.

N-Acetoxy-4-methoxy-2,2,6,6-tetramethyl-3-piperideine (1). A solution of toluenesulphonic acid monohydrate (684 mg, 3.6 mmol) in benzene (400 ml) was heated under reflux in a flask fitted with a Dean and Stark trap until no further water separated (ca. 30 min). The water was run off and a solution of the ketal (7) (14.4g, 56 mmol) in benzene (25 ml) was added to the toluenesulphonic acid solution. The mixture was heated under reflux for 30 min and the contents of the trap were run off twice during this period to remove entrained methanol. The mixture was then cooled to room temperature, washed with saturated aqueous $NaHCO_3$ (2 x 200 ml), dried (Na_2SO_4) and the solvent removed under reduced pressure. The residual oil was purified in batches by short-path distillation at 0.8 mm Hg (Kugelrohr, oven temperature 150°C) to give the enol ether (1) as a pale oil (11.5g; 91%) which solidified to a waxy solid, m.p. 37-40°C on standing at 4°C. Anal: Calc. for $C_{12}H_{21}NO_3$: M.W. 227.1522. Found: M^+ 227.1519.

EXAMPLE 2

2'-O,3'-O-(N-Acetoxy-2,2,6,6-tetramethylpiperidinylidene)adenosine monophosphate (10). AMP monohydrate (free acid form) (2.2g, 6 mmol) was suspended in dry dimethylformamide (50 ml) and the solvent was removed under vacuum (< 1 mm Hg) at a bath temperature of 35°C. The procedure was repeated a further three times in order to remove traces of water from the nucleotide, and at the end of the final cycle the residue was thoroughly pumped under vacuum to ensure complete removal of the dimethylformamide.

In a separate flask, toluenesulphonic acid monohydrate (5.7g; 30 mmol) was similarly dried by repeated vacuum evaporation from anhydrous acetonitrile (4 x 50 ml), then dissolved in anhydrous acetonitrile (440 ml) together with the enol ether (1) (20g, 88 mmol). This solution was added rapidly to the dried nucleotide and the resulting suspension was stirred for 7 days at room temperature. The undissolved fraction of the AMP was removed by filtration, the filtrate was diluted with 10 mM triethylammonium

bicarbonate (TEAB) buffer, pH 7.4 (2 l) and the mixture was extracted with petroleum ether (3 x 400 ml). The organic extracts were discarded and the aqueous solution was adjusted to pH 7.0 and applied to a DEAE-cellulose column (500 ml void volume) at a flow rate of 80 ml/h. When fully loaded, the column was washed with 10 mM TEAB, pH 7.4 until the absorbance (260 nm) of the effluent returned to zero, then eluted with a linear gradient formed from 10 and 250 mM TEAB, pH 7.4 (each 1.5 l). Fractions were collected in 13 ml aliquots and monitored by u.v. and analytical h.p.l.c. (Merck Lichrocart C8 column, mobile phase 45% MeOH - 55% 10 mM sodium acetate pH 6.5, flow rate 1.5 ml/min. Retention times for AMP and the spiroketal (10) were 1.5 and 3.3 min respectively). All fractions containing the spiroketal (10) were combined and concentrated under reduced pressure, then evaporated under vacuum with methanol several times to remove buffer salts. The product was further purified by reverse-phase preparative h.p.l.c. (Waters C_{18}, mobile phase 35% methanol - 65% 10 mM sodium acetate pH 6.5, flow rate 4 ml/min). Fractions of 16 ml were collected and assayed by analytical h.p.l.c. (see above) and those containing pure spiroketal (10) were combined and concentrated under reduced pressure to remove most of the methanol and finally desalted on a DEAE cellulose column as described above. The eluted material was freed of buffer salts as described to give the pure spiroketal (10)as its triethylammonium salt (1.2 mmol; 20%).

2'-O,3'-O-(N-Acetoxy-2,2,6,6-tetramethylpiperidinylidene)adenosine triphosphate (11). The AMP spiroketal (10) (120 umol) was pyrophosphorylated by known procedures[2,3] and purified by ion-exchange chromatography on DEAE-cellulose as described above, using a column of void volume 150 ml, a linear gradient formed from 10 and 350 mM TEAB (each 600 ml) and a flow rate of 50 ml/h. Fractions were analysed by reverse-phase h.p.l.c. (Conditions as above). The retention time for the ATP spiroketal

(11) was 1.83 min). Fractions containing pure product were combined and freed from buffer salts as above to afford the ATP spiroketal acetate (11) as its triethylammonium salt (57 µmol; 47%).

2'-O,3'-O-(N-Oxyl-2,2,6,6-tetramethylpiperidinylidene) adenosine triphosphate (12). To a solution of the spiroketal (11) (400 µmol) in 50% aqueous methanol (62 ml) was added a solution of KOH (1.34g) in methanol (21 ml), and the mixture was left at room temperature and open to the atmosphere. The reaction was monitored by h.p.l.c. (Merck Lichrocart C8, mobile phase 12% MeCN - 88% 50 mM KH_2PO_4 pH 5.5, flow rate 1.5 ml/min. Retention times for the spiroketal (11) and the ATP spin label (12) were 4.0 and 13.0 min respectively). When the hydrolysis/oxidation reaction was complete (48 h), the reaction mixture was neutralised with 1M HCl (24 ml) and diluted with 10 mM TEAB (600 ml). The pH was adjusted to 7.0 and the solution was applied to a DEAE-cellulose column (void volume 400 ml) at a flow rate of 80 ml/h. The column was eluted with a linear gradient formed from 10 and 350 mM TEAB (each 2 l) and fractions were analysed by h.p.l.c. Fractions containing the product were seen to contain an impurity (approx. 3%; h.p.l.c. retention time 1.2 min) which was identified as free ATP. These fractions were combined and freed from buffer salts as above. A portion of the contaminated spin-labelled product (100 µmol) was further purified by preparative h.p.l.c. (Waters C_{18}, flow rate 4 ml/min). The preparative column was eluted first with 10 mM sodium acetate, pH 6.5 until all the free ATP had eluted, then with 5% MeCN - 95% 10 mM sodium acetate, pH 6.5. Fractions containing the pure spin label (12) (90 µmol)

were pooled, desalted on DEAE-cellulose as described above and stored at -20°C either as the triethylammonium salt or after treatment with Dowex 50 (Na^+ form) as the sodium salt.

EXAMPLE 3

2'-O,3'-O-(N-Acetoxy-2,2,6,6-tetramethylpiperidinylidene)-5'-O-benzoyluridine (13). 5'-O-benzoyluridine (350 mg, 1 mmol) was added to a solution of toluenesulphonic acid monohydrate (190 mg, 1 mmol) in dry tetrahydrofuran (5.6 ml), followed by addition of N-acetoxy-2,2,6,6-tetramethyl-3-piperideine (2.2 g, 9.7 mmol). The solution was left at room temperature for 7 days, then diluted with ethyl acetate (100 ml) and washed with saturated $NaHCO_3$, dried (Na_2SO_4) and evaporated. The oily residue was purified by flash chromatography (Merck 9385 silica gel) using ethyl acetate - petroleum ether (3:2) as the eluting solvent to give the title product (13) as a pale gum (311 mg; 57%). The material was homogeneous by thin layer chromatography on silica gel (ethyl acetate-light petroleum 3:2; R_f 0.35) and spectroscopic properties (IR, ^1H and ^{13}C NMR, UV) were in conformity with its structure.

References

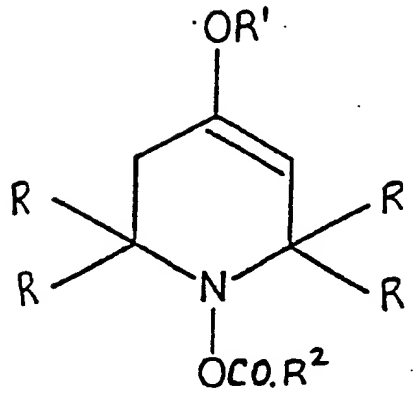
1. C.M. Paleos and P. Dais, J. Chem. Soc. Chem. Commun., 1977, 345-346.

2. D.E. Hoard and D.G. Ott, J. Am. Chem. Soc., 1965, 87, 1785-1788.

3. M. Maeda, A.D. Patel and A. Hampton, Nucleic Acids Res., 1977, 4, 2843-2853.

CLAIMS

1. A compound of the formula:-

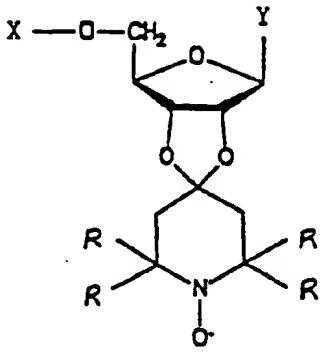


(1)

wherein R represents a CH_3 or CD_3 group;
R^1 represents an alkyl group;
R^2 represents an alkyl or aryl group; and
N may be either an ^{14}N or ^{15}N atom,
with the proviso that when R represents CD_3, the methylene and vinyl hydrogen atoms of the six-membered ring are deuterium.

2. A compound as claimed in claim 1, wherein each of R, R^1 and R^2 is methyl.

3. A compound of the formula:-



(2)

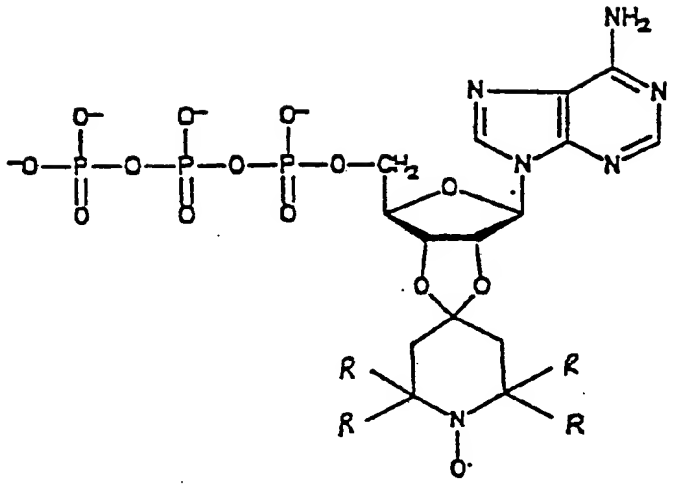
wherein R represents a CH_3 or CD_3 group;

N may be either an ^{14}N or ^{15}N atom;

X represents hydrogen or a mono-, di- or triphosphate group, or a phosphate ester derivative; and

Y represents a purine or pyrimidine base, with the proviso that when R represents CD_3, the methylene hydrogen atoms of the six-membered piperidine ring are hydrogen or deuterium.

4. A compound as claimed in claim 3 of the formula:-



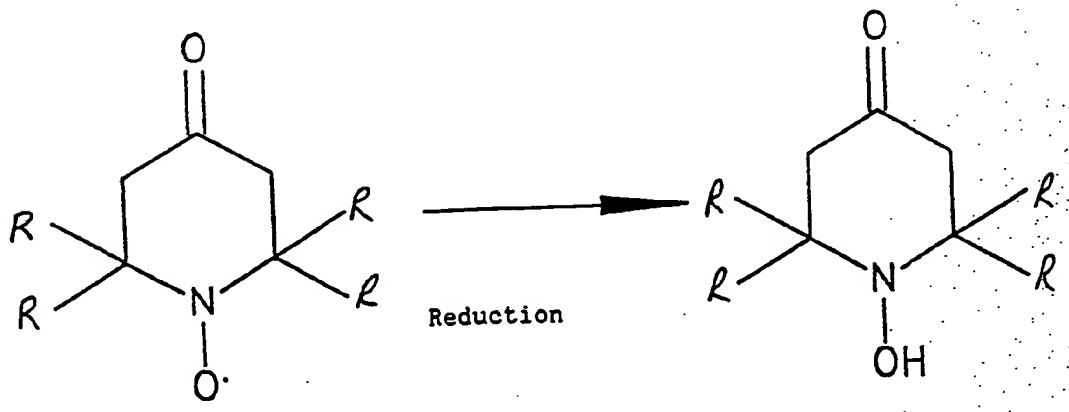
(3)

wherein R is as defined in claim 3.

5. A compound as claimed in claim 4, wherein R is methyl.

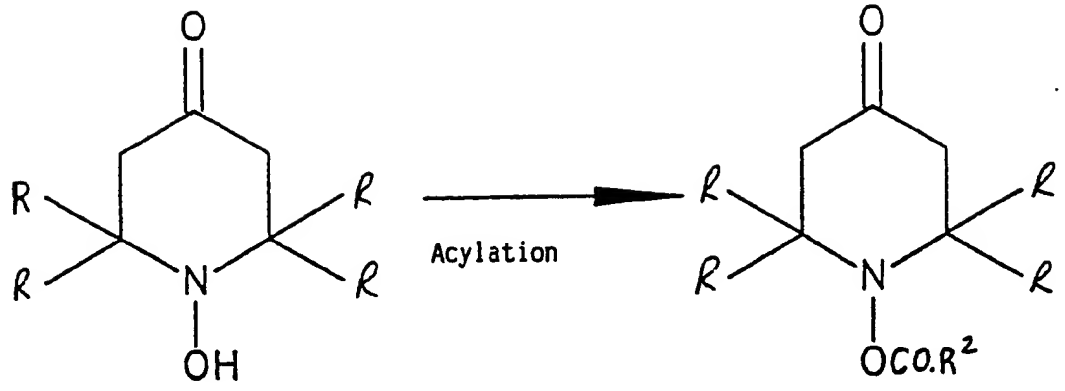
6. A method for preparing a compound as claimed in claim 1, which comprises the following sequence of reactions:-

i)



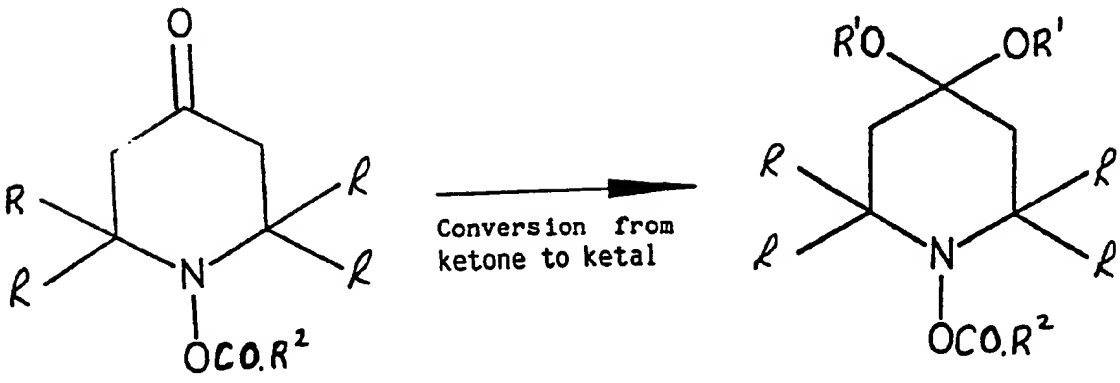
(4) (5)

ii)


O
R
R
R
R
N
OH
Acylation
O
R
R
R
R
N
OCO.R²

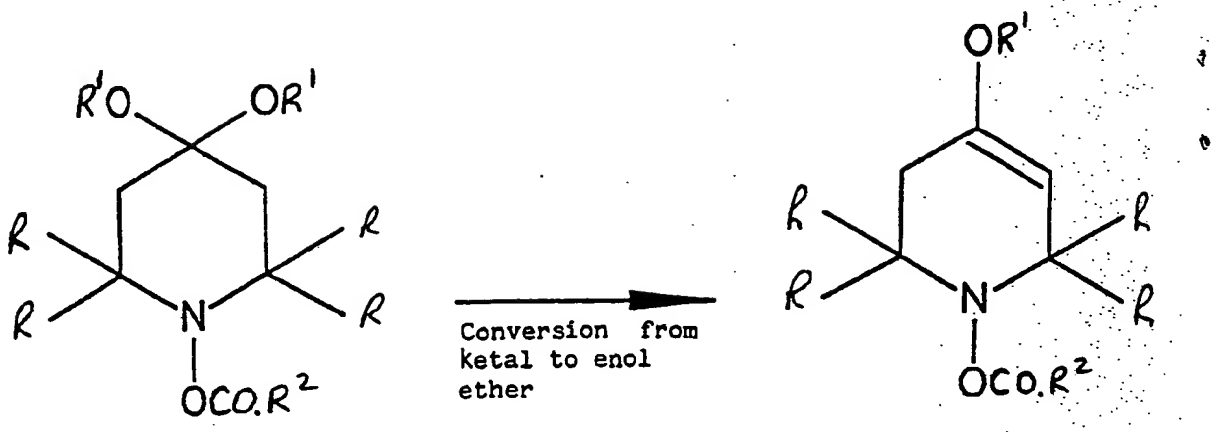
(5) (6)

iii)


O
R
R
R
R
N
OCO.R²
Conversion from ketone to ketal
R'O
OR'
R
R
R
R
N
OCO.R²

(6) (7)

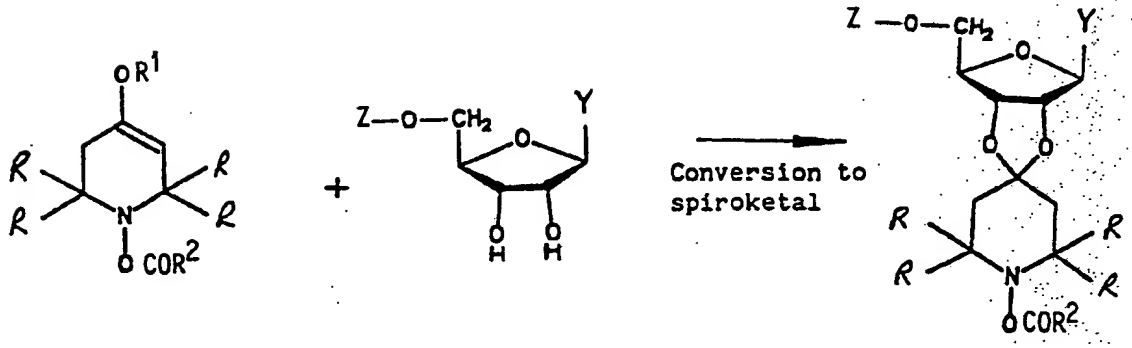
iv)



(7) (1)

7. A method for preparing a compound as claimed in claim 3, which comprises the following sequence of reactions:-

v)



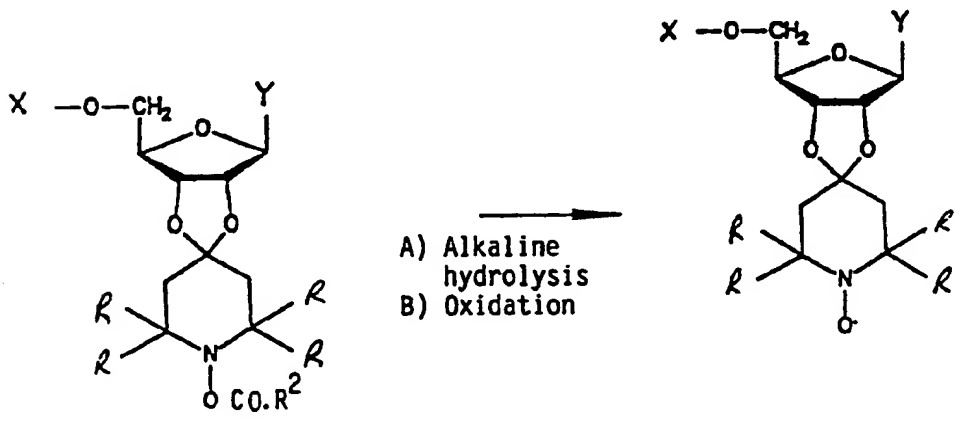
(1) (8) (9)

wherein Z is a protecting group for the 5'-hydroxyl position and which may either equate to group X (as hereinbefore defined, except when X represents hydrogen) or be capable of conversion or removal to leave a group X in that position; and

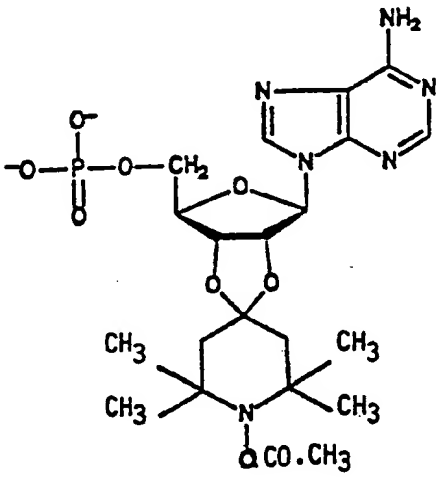
R, R^1, R^2 and Y are as previously defined, with the further proviso that when R represents CD_3, the 2'- and 3'-hydroxyl hydrogen atoms in compound (8) may be deuterium in order that all four methylene hydrogen atoms of the six-membered piperidine ring of compound (9) are deuterium;

vi) either a) where group Z equates to group X, optionally further phosphorylating the compound (9) at that position, or b) treating the compound (9) such that Z is removed, and optionally phosphorylating, to leave a group X at that position; and

vii)

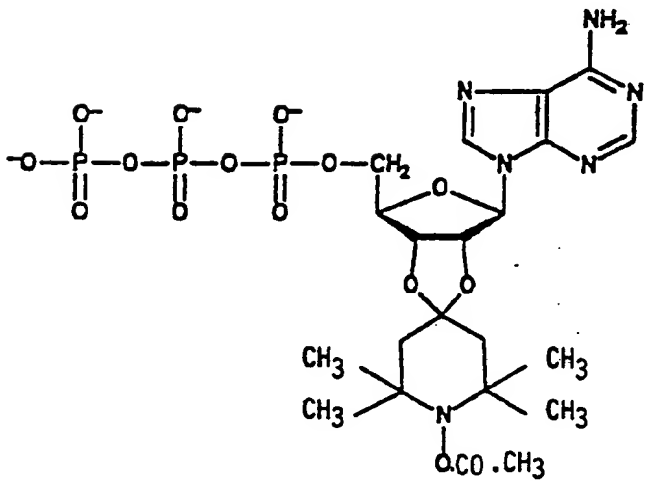


8. A method as claimed in claim 7, wherein step v) comprises reacting compound (1) with adenosine 5'-monophosphate to produce a compound of the formula:-



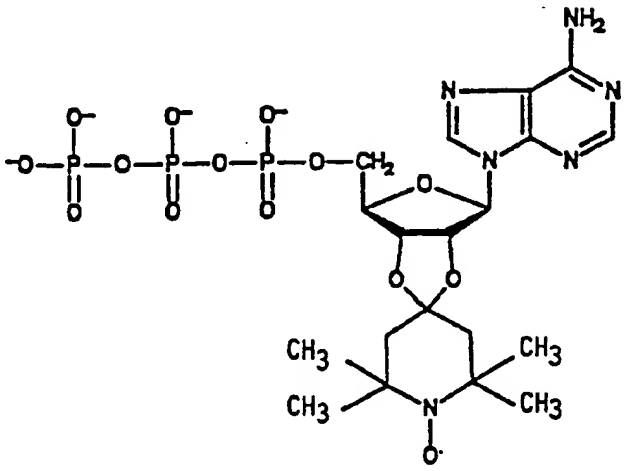
(10)

step vi) comprises pyrophosphorylating the product of step v) to produce a compound of the formula:-



(11)

and step vii) comprises deacetylation and air oxidation of the product of step vi) to produce a compound of the formula:-



(12)

9. Use of a compound as claimed in claim 3 as a spin labelled probe or as a part of such a probe.

10. A method of investigating or determining protein orientation or structure which comprises the use of a compound as claimed in claim 3 or claim 4.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/01146

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 07 D 211/94 C 07 H 19/20 C 07 H 19/04

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 B 61/00 C 07 D 211/00 C 07 H 19/00

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	FR,A,2235103 (COMMISSARIAT A L'ENERGIE ATOMIQUE) 24 January 1975, see pages 7-10; compounds I-IV	1-10
A	EP,A,0133674 (SCHERING) 6 March 1985, see claims; page 4	1-10

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15-10-1991	27. 11. 91
International Searching Authority: EUROPEAN PATENT OFFICE	Signature of Authorized Officer: Maria TORIBIO

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9101146 SA 49533

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 31/10/91 The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR-A- 2235103	24-01-75	None	
EP-A- 0133674	06-03-85	DE-A- 3328365	21-02-85
		CA-A- 1230114	08-12-87
		JP-A- 60094960	28-05-85
		US-A- 4925652	15-05-90
		US-A- 4845090	04-07-89